MTHFR C677T Detection Kit v1

USER MANUAL

For *in vitro* Diagnostic Use

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1. PRODUCT DESCRIPTION

Bosphore® MTHFR C677T Detection Kit v1 detects MTHFR mutation; namely C677T (a change of cytosine nucleotide with the thymine at position 677) in human biological samples. Wild type MTHFR allele is amplified and fluorescence detection is accomplished using the Cy-5 filter. Allele with MTHFR polymorphism is amplified and fluorescence detection is accomplished using the FAM filter.

2. CONTENT

Bosphore® MTHFR C677T Detection Kit v1 is composed of Real-Time PCR reagents.

Component	REAGENT	100 Tests	50 Tests	25 Tests
1	dH_2O	(1000 µl)	(1000 µl)	(1000 µl)
2	PCR Mix	(1375 µl)	(688 µl)	(344 µl)
3	Detection Mix1	(100 µl)	(50 µl)	(25 µl)
4	Positive Control	(30µl)	(15 µl)	(15 µl)

3. STORAGE

Bosphore® MTHFR C677T Detection Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min and the detection mix components should not be exposed to light more than 1-2 min. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

4. REQUIRED MATERIALS AND DEVICES

- Montania® 483 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and Cy5 filters (iCycler, iQ5, CFX–BioRad, LightCycler 1.5, 2.0, 480-Roche, 7500 Real-Time PCR System-ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Genomic DNA Whole Blood Kit (Anatolia Geneworks),or other high quality genomic DNA extraction kits/systems
- Deep freezer (-20°C)

- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

Important!:

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20°C.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- All the biological wastes produced during the nucleic acid isolation step; including the blood samples and material contacted with them, should be discarded into medical waste and disposed safely.

6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

7. POLYMORPHISM

MTHFR stands for Methylene-Tetra-Hydro-Folate-Reductase which is a key enzyme involved in amino acid metabolism of the body. It catalyzes the conversion of 5, 10-methylenetetrahydrofolate to 5 methyltetrahydrofolate, a cosubstrate for multistep process in the conversion of the amino

acid homocysteine to amino acid methionine which is then used to make proteins and other important compounds. [1] When there are genetic mutations (mostly single amino acid substitutions) occurred in the MTHFR gene, inhibiting the production of this enzyme, it may result in excess *homocysteine* level (hyperhomocytenemia) in blood plasma which is thought to be the susceptibility of health problems (occlusive vascular disease, neural tube defects, colon cancer, acute leukemia, methylenetetrahydrofolate deficiency) associated with homocystinuria.[2]

About 24 mutations in the MTHR gene have been identified in people with homocystinuria. Within 24 mutations, there are two variants, C677T and A1298C single nucleotide polymorphisms (SNP), are relatively common in many populations worldwide. The C677 variant nucleotide replaces cytosine with the thymine at position 677 in the MTHR gene which alters methylenetetrahydrofolate reductase and reduces its activity at higher temperatures (thermolabile) resulting in elevated levels of homocysteine in blood and homocystinuria associated health problems. [3]

8. METHOD

Bosphore® MTHFR C677T Detection Kit v1 is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. Primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore, Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual-labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The test utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hydrolysis probe during the extension phase. The probe is labeled at the 5′ end with a fluorescent reporter, and at the 3′end with another fluorescent molecule that acts as a quencher for the reporter. When the two fluorophores are in close proximity, even if the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step, Taq Polymerase encounters and cleaves the probe bound to the template. As the reporter is relieved from the suppressing effect of the quencher, fluorescence signal can be detected.

As the PCR product accumulates, the fluorescence generated by the reporter increases. The point at which the signal rises above background level and becomes detectable, is called the threshold cycle (C_T).

Bosphore MTHFR C677T Detection Kit v1 employs multiplex PCR. MTHFR DNA, whether wild-type or polymorphic, is amplified in a single reaction, using sequence-specific primers against mutant and wild-type alleles. The fluorescent signal generated by the MTHFR polymorphism is detected by a probe labeled at the 3' end with FAM, through the FAM channel. In contrast, the fluorescent signal generated by the wild-type allele amplification, is detected by a second probe (labeled at the 5' end with a different reporter molecule, Cy5) through the Cy5 channel.

9. PROCEDURE

9.1. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Genomic DNA Whole Blood Kit (Anatolia Geneworks) isolation system is used with Bosphore® MTHFR C677T Detection Kit v1. The DNA isolation should be performed according to the manufacturers' instructions. The starting volume is 400 µl; the elution volume is 60 µl.

9.2. Kit Components

9.2.1. PCR Mix

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into E.Coli. The enzyme is provided in an inactive form. It is activated by a 15-minute 95 °C incubation step. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. PCR Buffer: contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20°C). dNTP Mix: Contains ultrapure quality dATP, dGTP, dCTP ve dTTP/dUTP.

9.2.2. Detection Mix

Detection Mix 1 contains MTHFR gene -specific forward and reverse primers and two dual-labeled probes against wild-type and mutant MTHFR allele.

9.2.3. Positive Control

The positive control contains heterozygous MTHFR gene containing one copy of the wild-type and mutant MTHFR allele each. It should be included in the PCR to efficiently test whether the samples being analyzed are positive or negative. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

9.3. Preparing the PCR

Positive control should be added into the PCR reaction together with the samples and the negative control (PCR-grade water). Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 3 samples, an extra 10% should be added to the total sample number.

PCR Mix	12.5 µl
Detection Mix 1	0,9 µl
dH2O	8,6 µl
Sample DNA	
Positive/Negative Control	3 µl
Toplam Hacim	25.0 µl

Pipette 22 µl of the master mix into the PCR tubes or strips, and add 3 µl of DNA (sample/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

9.4. Programming the Montania® 483 Real-Time PCR Instrument

The thermal protocol for Bosphore® MTHFR C677T Detection Kit v1 is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

Initial denaturation	95°C	14:30 min.	
Denaturation	95°C	00:30 min.	35 cycles
Annealing and Synthesis (Data Collection)	65°C	00:30 min.	
Hold	22°C	05:00 min.	

Montania® 483 Real-Time PCR Instrument is installed and calibrated as it is delivered to the end user.

In order to establish an appropriate link between the system components, first the thermal cycler and the optical module, and then the PC and the software should be started.

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and Cy5),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol.

These steps are described below:

From the main menu of the Montania® 483 Real-Time PCR Instrument, “File” and then “New” is selected. “Create a new Experiment” is selected. In the “Select Channel” window channels 1 (FAM) and 3 (Cy5) are selected (Fig. 1). Samples, positive and negative controls are identified in the “Module Edit” menu (Fig. 2). To select the thermal protocol “Gene Amplification” menu is used. The “Open” button in the “Experiment Program” is clicked and the appropriate thermal protocol is selected. (Fig. 3a). The thermal cycles of the selected protocol is displayed. The experiment starts by clicking the “Start” button (Fig. 3b).

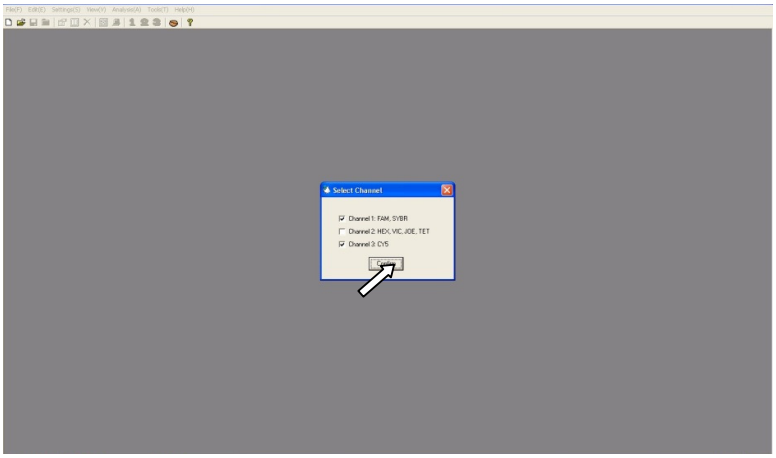

Fig. 1: Filter Selection in Montania® 483

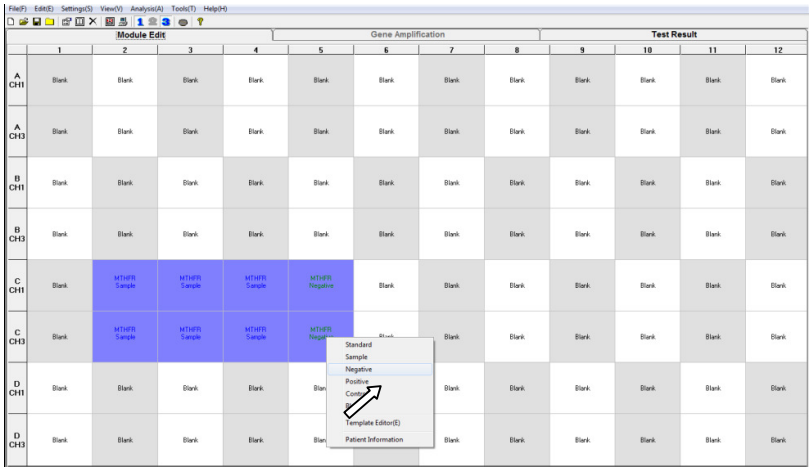

Fig. 2: Sample Location and Identification

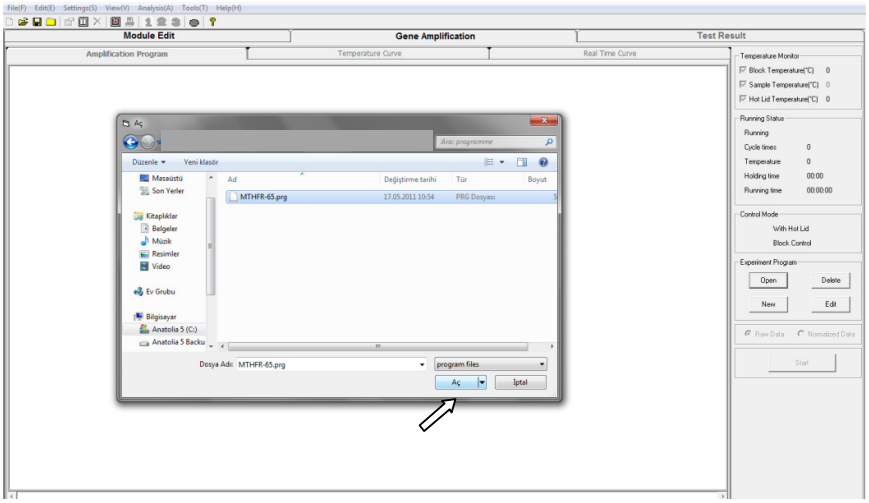

Fig. 3a: Selecting the Thermal Protocol

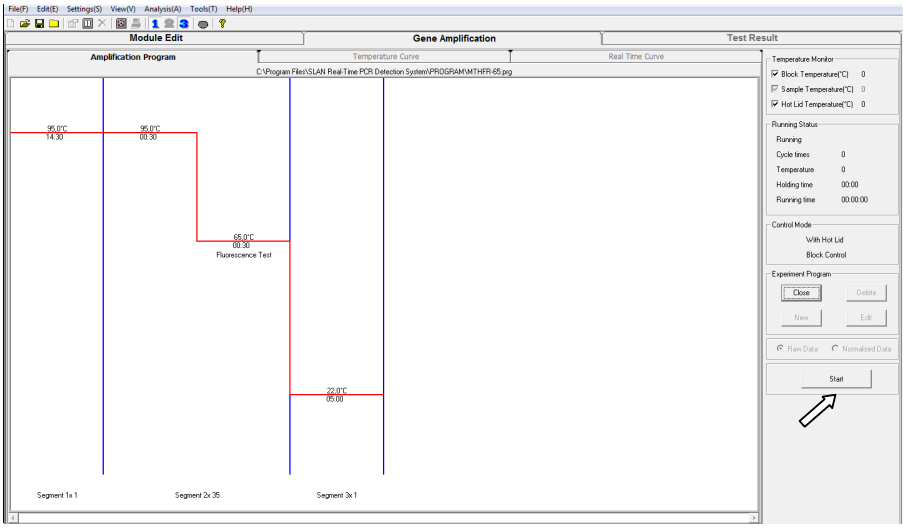

Fig. 3b: Starting the Experiment

10. ANALYSIS

By the end of the thermal protocol, the Montania® 483 Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 4.

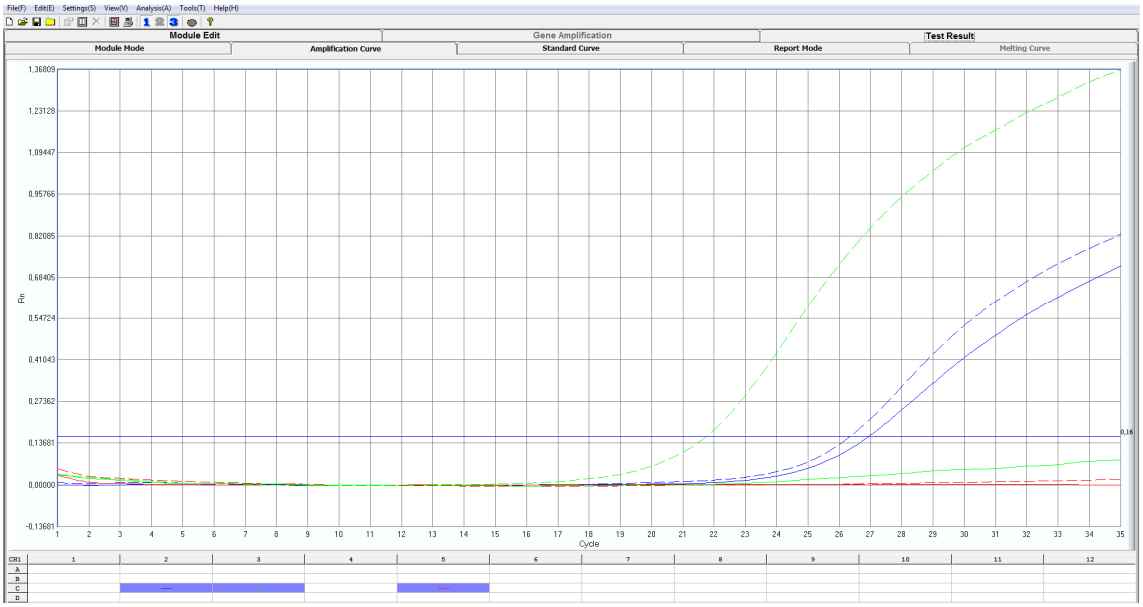

Fig. 4: Amplification Curve of a Bosphore® MTHFR Detection v1 test

Analysis of the results should be performed by trained personnel who have received the required training for analysing Real-Time PCR data. We recommend that the test results must be evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, the system allows pulling down the threshold as much as possible in order to detect slight amplifications. In this case, attention should be paid to keep the threshold line above the background.

Amplification should be observed from both FAM and Cy5 filters of the heterozygous positive control during the test. The amplification of both filters from the tested samples should be compared with that of the positive control. Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if impairment in the product's performance is observed (See the last page for contact information).

The qualitative results of the test are displayed on the "Report Mode" screen. The samples that cross the threshold in Channel 1 (FAM) and Channel 3 (Cy5) are displayed as positive, for polymorphic and wild-type alleles respectively.

The following table shows the possible results and their interpretation:

Signal in both FAM and Cy5	Heterozygous polymorphic: The sample has both wild-type and polymorphic MTHFR alleles.
No signal in FAM, signal in Cy5	Homozygous wild-type: The sample has only wild-type MTHFR allele

Signal detected only in the FAM filter	Homozygous polymorphic: The MTHFR DNA in the sample is composed of polymorphic alleles
No signal in FAM and Cy5	The test should be repeated

11. TROUBLESHOOTING

Please contact the manufacturer in case of a problem during a run.

Signal from FAM/Cy5 Filter in the Negative Control

Contamination	Use filter-tips. Repeat PCR with new kit components.

The Threshold is Above Low Signals

The threshold should be manually adjusted	Using the mouse pull the threshold down until it cuts the low signals. Avoid the background and the signal from negative control.

12. REFERENCES

1. Goyette, Sumner, Milos, et al., Human methylenetetrahydrofolate reductase: isolation of cDNA, mapping and mutation identification, Nature Genetics, 1994. 7(2): p.195-200

2. Genetic home reference, NIH Resources, MTHFR, January 2008

3. Sibani S, Christensen B, O'Ferrall E, Saadi I, Hiou-Tim F, Rosenblatt DS, Rozen R (2000). "Characterization of six novel mutations in the methylenetetrahydrofolate reductase (MTHFR) gene in patients with homocystinuria". *Hum. Mutat.* 15 (3): 280–7.

13. SYMBOLS

Use by

Lot/Batch

Catalog number

Temperature limitation

Caution, consult accompanying documents

Manufacturer

In Vitro Diagnostic Medical Device

14. CONTACT INFORMATION

Anatolia Tanı ve Biyoteknoloji A.Ş.

Egitim Mh. Kasap Ismail Sk.
No:10/23 Kadikoy 34722
ISTANBUL-TURKEY
Phone: +90 216 330 04 55
Fax: +90 216 330 00 42
E-mail: info@anatoliageneworks.com
www.anatoliageneworks.com

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